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(54) Title: MACROPOROUS CROSS-LINKED POLYMER PARTICLES

(57) Abstract: The present invention relates to a method of producing one or more macroporous cross-linked polymer particles, which comprises polymerisation and cross-linking of divinyl ether monomers in an inert solvent that comprises ether groups, in which method the polymerisation is free radical initiated. The invention also relates to particles so produced, which are useful as separation medium e.g. in RPC or, after suitable derivatisation, in other chromatographic methods.

MACROPOROUS CROSS-LINKED POLYMER PARTICLES

Technical field

The present invention relates to the production of macroporous cross-linked polymeric particles useful as packings or carrier of ligands in chromatographic methods as well as to macroporous cross-linked polymeric particles as such.

Background

There are numerous methods of chromatography available today, such as ion exchange chromatography (IEX), hydrophobic interaction chromatography (HIC), reversed phase chromatography (RPC), affinity chromatography, gel filtration etc. The feature that distinguishes chromatography from most other physical and chemical methods of separation is that two mutually immiscible phases are brought into contact wherein one phase is stationary and the other mobile. The sample mixture, introduced into the mobile phase, undergoes a series of interactions i.e. partitions many times between the stationary and mobile phases as it is being carried through the system by the mobile phase. Interactions exploit differences in the physical or chemical properties of the components in the sample. These differences govern the rate of migration of the individual components under the influence of a mobile phase moving through the stationary phase. Separated components emerge in a certain order, depending on their interaction with the stationary phase. The least retained component elutes first, the most strongly retained material elutes last. Separation is obtained when one component is retarded sufficiently to prevent overlap with the zone of an adjacent solute as sample components elute from the column.

Chromatographic separation methods are useful e.g. for recovery of biomolecules, such as nucleic acids and proteins, and small organic molecules from liquids. In addition, chromatographic separation methods can also be used for recovery of liquids, in which case impurities such as organic or inorganic molecules are removed to result in a liquid of higher purity.

The matrix used in chromatography is composed of a carrier material, which is usually in the form of particles, monolithic matrices or the like. In some applications, such as RPC, the surface of the carrier itself will provide the interaction with the target molecules. In other applications, such as ion exchange or affinity chromatography, the carrier has been provided with ligands that comprise charged groups or affinity groups for interaction with target molecules. In either case, the carrier materials used can be classified as inorganic materials, such as silica, and organic material, including the groups of synthetic polymers, such as poly(divinylbenzene) (DVB) and poly(divinylbenzene/styrene), and native polymers, such as agarose.

Silica has been used for several years as chromatography beads. However, a general problem with silica is that it is susceptible to hydrolysis in basic conditions, and accordingly the conventional cleaning in place (cip) procedure using sodium hydroxide is not suitable in this case. For the same reason, silica matrices cannot be operated in chromatography under high pH conditions, which severely limits their use in a wide range of applications. More recently, alkyl-bonded silica particles have been suggested for reversed phase liquid chromatography. However, alkyl-bonded silica contains residual silanol groups or impurities, which in some cases results in peak tailing and a poor chromatographic performance in general.

Matrices based on native organic polymers, such as polysaccharides and especially agarose, are used for many chromatographic applications, especially in a derivatised form since the hydroxyl groups available on their surfaces render them easy to derivatise. Agarose particles can be cross-linked by addition of a chemical cross-linker, and they will become porous as a result of the gelling procedure. The nature of such particles is what is generally denoted gelporous, and such gelporous particles are in general less advantageous in procedures where high pressures are used.

Matrices based on synthetic organic polymers, such as divinylbenzene (DVB) and styrene, on the other hand are tolerant to cleaning in place, but their aromatic groups can sometimes cause undesired effects in chromatographic methods. More specifically, if

such a matrix is used to adsorb molecules that also comprise aromatic groups, an interaction between their respective π electrons will occur, resulting in peak tailing and long retention times. A disadvantage of DVB based matrices is that they often exhibit a large proportion of undesired micropores, which results in impaired mass transfer properties in chromatography. It has been hypothesised that these micropores, which are especially disadvantageous in RPC, are produced as a result of the rapid polymerisation of DVB monomers in suspension polymerisation. Another disadvantage of styrene based matrices appears in cases where it is desired to derivatise the surface of the particle to change its properties, since this kind of matrices exhibit a chemical inertness that in most cases necessitates several surface modification steps before the desired change can be achieved. A further disadvantage of organic polymer-based polymers such as styrene and DVB is that they are somewhat compressible at the high-pressure conditions that are used in high-performance separation methods.

In order to provide improvements to the above-discussed groups of matrix materials, newer alternatives have been presented. For example, in order to produce improved preparative ion exchange chromatography matrices of improved mechanical and chemical stability, Britsch et al (Recovery of Biological Products) have suggested a copolymer synthesised of a mixture of a hydrophilic vinyl ether and a bifunctional acrylamide monomer. The resulting product is accordingly based on amide type cross-linking. However, as is well known, acrylamide requires care to be taken when handled to avoid negative health effects, which will result in a more cumbersome and inconvenient process. Furthermore, the hydrolysis sensitivity of amides in general may render this kind of matrices less advantageous for certain applications.

Ericsson et al (WO 95/13861) have disclosed a separation method, wherein hydrophilic vinyl ether polymers attached to a support are used as a matrix. More specifically, the polymers suggested therein comprise a poly(vinyl ether) chain comprising identical or different vinyl subunits, and the support is illustrated with agarose particles. The polymers are synthesised using cationic polymerisation.

Further, in order to avoid the disadvantages of alkyl-bonded silica matrices and polymer matrices in reversed phase liquid chromatography, Hirayama et al (Chromatographia Vol. 33, No 1/2, January 1992, 19-24) suggest suspension copolymerisation of an alkylvinyl ether with triethylene glycol divinyl ether. Thus, in this method, the organic phase was comprised of two different kinds of reactive vinyl ether monomers, the mutual ratio of which was used to set a specific hydrophobicity of the resulting product.

US patent number 5,334,310 discloses a liquid chromatographic column that contains a separation medium in the form of a macroporous polymer plug, also known as a monolith. The polymerisation mixture from which the plug is prepared contains at least one polyvinyl monomer, a free radical generating initiator and a porogen. The so prepared plug contains small pores of diameters less than about 200 nm as well as large pores of diameters greater than about 600 nm. Such a large pore diameter range is advantageous for monoliths to allow a high flow rate, but clearly above the useful pore diameter ranges in polymer particles intended for chromatography.

EP 1 179 732 presents a solution as regards how to produce a polymer adsorbent that exhibits selected porosity and permeability characteristics. This has been achieved by a method, preferably a suspension polymerisation method, wherein selected mixed porogens in selected proportions relative to the monomer are used. The thereby produced adsorbent is due to its rigidity especially suitable for use as an RPC stationary phase at high-pressure conditions.

Accordingly, since the various groups of chromatographic matrix materials exhibit certain advantages and disadvantages, it is often the case that the matrix used is selected depending on the kind of target molecule, the purpose of the separation etc. Alternatively, two or more different chromatographic principles and/or different matrix materials are sometimes combined into sequence of chromatographic steps, often denoted polishing, capture etc. Thus, each separation principle can be viewed as one tool useful in a toolbox, where there is a constant need of new tools. Accordingly, despite the known matrix

materials discussed above, there is still a need of novel methods to use as supplement, i.e. as further tools in a tool box.

Summary of the present invention

Accordingly, one object of the present invention is to provide a novel material useful as a matrix in chromatographic separation methods, which exhibits properties that differ from known materials. This is achieved by providing macroporous cross-linked polymer particles, which have been polymerised from divinyl ether monomers in an inert solvent that comprises ether and/or polyether groups.

Another object of the invention is to provide such polymer particles, which result in novel selectivity patterns when used as a matrix in chromatography, such as RPC for protein separation.

Yet another object of the invention is to provide polymer particles as described above, the surfaces of which are easily derivatised e.g. with functional groups for ion exchange or affinity chromatography, with hydrophobic groups for hydrophobic interaction chromatography (HIC) or any other groups that change the properties of the particle surface in a desired way. This is achieved by providing macroporous cross-linked polymer particles that exhibit a sufficient number of vinyl groups available for reaction on their surfaces.

Another object of the invention is to provide polymer particles essentially devoid of micropores with a narrow pore size distribution.

Another object of the invention is to provide polymer particles wherein the average pore size has been carefully controlled. This is achieved by using polyethers with different molecular weights as the inert solvent in the method according to the invention.

One more object of the invention is to provide polymer particles as described above, that fulfil one or more of the criteria above and at the same time exhibit an advantageous me-

chanical stability and consequently withstand high pressures when packed in chromatographic columns.

One more object of the present invention is to provide a method of producing a novel material as mentioned above, in which method the average pore size of the final product is carefully controlled. This is achieved by radical polymerisation of divinyl ether monomers in an inert solvent that comprises ether and/or polyether groups of suitable molecular weights, where the solvent also acts as a porogen.

An additional object of the present invention is to provide a method of producing macroporous cross-linked polymer particles, wherein the polymerisation rate is slower than for prior art polymerisations of divinyl benzene and styrene. This can be desired for various reasons, one of which is that it appears to result in a macroporous structure with less micropores.

One or more of the above-defined objects can be achieved as defined in the appended claims. Further objects and advantages of the invention appear from the detailed description that follows.

Brief description of the drawings

Figure 1 shows separation of 4 different peptides on an underivatised macroporous polymer particle and a macroporous polymer made more hydrophobic by chemical modification with octadecyl vinyl ether.

Figure 2 shows the separation of 6 proteins and illustrates the difference in selectivity between divinyl ether particles according to the invention and prior art DVB/Styrene particles.

Figure 3A-C show the pore size distributions of three macoporous polymer particles of the present invention.

Definitions

The term "chromatography" is used herein to include dynamic as well as batch procedures.

The term "matrix" is used herein to denote a material that is useful as a stationary phase in chromatography. Accordingly, e.g. an RPC matrix will normally be comprised of one material throughout each bead, while an ion exchange matrix will denote a carrier provided with ligands that comprise functional groups for binding target molecules.

The term "macroporous" particles means particles that are porous both in a dry or wet state, as compared to gelporous particles that are porous only in the wet state.

The term "particle" means herein a chromatographic carrier of essentially spherical shape .The term "divinyl ether" means herein a bifunctional vinyl ether, wherein the two functions are two vinyl groups. Thus, in the context of vinyl ethers, the term bifunctional refers to the function of an unsaturated carbon-carbon bond.

The term "porogen" refers to an inert solvent (low molecular weight or polymeric) present during the polymerisation reaction that gives rise to the formation of a macroporous polymer at some stage during the polymerisation.

The term "inert" means being unreactive, i.e. not capable of participating in a chemical reaction such as polymerisation.

The term polyether refers to a polymer comprising one or more ether groups with the general formula (I)

$$X\text{–}(O\text{-}R\text{-}O\text{-})_n\text{-}Y \qquad (I)$$

where X and Y may, or may not be identical end groups, for instance alkyl, aryl or hydrogen groups, for example hydrogen, methyl, ethyl, tert-butyl or phenyl groups. R is an alkyl or aryl group, linear or branched, which may contain other functional groups, (e.g. carboxylic acid groups, hydroxyl groups or amines, etc) and n is an integer between 2 and 10000, such as between 3 and 5000, or preferably between 5 and 100.

Detailed description of the invention

A first aspect of the present invention is a method of producing one or more macroporous cross-linked polymer particles, which comprises polymerisation and cross-linking of divinyl ether monomers in an inert solvent that comprises ether groups, in which method the polymerisation is free radical initiated. The solvent can comprise ethers as well as polyethers, for example as mixtures. Thus, in one embodiment, the solvent comprises polyether groups. In another embodiment, the solvent is a mixture of ether and/or polyether groups and an alcoholic solvent, preferably comprised to a substantial proportion of ether and/or polyether groups.

The macroporous cross-linked polymer particles according to the invention polymers can for example be prepared according to the basic principles of conventional suspension polymerisation (see e.g. J. R. Benson and D. J. Woo, J. Chromatographic Sci., 1984, 22, 386). In brief, an organic solution comprising the desired divinyl ether monomers is mixed with an ether solvent (also denoted porogen in this context) and a thermal initiator and contacted with an aqueous phase containing water, an emulsifying agent and optionally other additives. The organic phase and the aqueous phase will form a suspension that is stirred, preferably mechanically, until the desired droplet size is obtained. The suspension is then heated to an elevated temperature, typically 65 °C, which will lead to thermal decomposition of the initiator and thereby to initiation of the polymerisation. The suspension will be kept at an elevated temperature until the polymerisation is considered to be complete, which may typically occur after about 24 hours. The resulting macroporous particles can easily be separated from the aqueous phase e.g. on a sintered glass filter, and subsequently be washed with water and/or organic solvents to remove the emulsifier and the porogen, respectively.

Accordingly, the divinyl ether monomers will act as monomers during the polymerisation as well as as cross-linkers. As will be discussed in more detail in the experimental part below, a deliberate choice of a solvent of a small or large molecular weight will result in a correspondingly small or large average pore size. Thus, in a specific embodiment, the

present invention also provides a different solution to the problem addressed e.g. by the above discussed EP 1 179 732.

In the most advantageous embodiment of the present method, the polymerisation is free radical initiated. The free radical initiator can be any suitable commercially available initiator, based e.g. on diacetyl peroxide, dibenzoyl peroxide, dilauroyl peroxide, t-butyl peroxypivalate, t-butyl peroctoate, t-amyl peroxypivalate, t-butyl peroxy-2-ethylhexanoate, di-(4-t-butylcyclohexyl)peroxydicarbonate, 2,2'-azobis(isobutyronitrile), 2,2'-azo-bis(2,4-dimethylvaleronitrile), or 1,1'-azo-bis(cyanocyclohexane), and mixtures thereof. Specific examples are the products V65 (=2,2'-azo-bis(2,4-dimethylvaleronitrile, Wako Pure Chemical Industries) or AIBN (=2,2'-azobis(isobutyronitrile), from Acros Organics). The choice of the free radical initiator is easily made by the skilled in this field considering the other conditions and reagents used, which will be discussed in more detail below. The amount thereof is not critical, as long as it is considered that too large an excess thereof might have a negative impact on the polymerisation, while a too small amount may not be sufficient for the desired result. Accordingly, an illustrative amount of radical initiator is within the range of 1-10 mole% (per monomer), such as 1-2 mole%.

The present invention describes for the first time a method of producing cross-linked macroporous polymer particles by polymerisation of divinyl ethers in an inert solvent comprising one or more ethers. Even though divinyl ethers have been polymerised for other purposes, such as in surface coatings application, their advantages as cross-linkers in methods to provide macroporous polymer particles have not been fully appreciated before. Vinyl ethers can successfully be polymerised by a cationic mechanism to yield high molecular weight polymers. On the contrary, vinyl ethers polymerise poorly by a free radical mechanism in traditional low-molecular weight solvents. For this reason, vinyl ethers have not to this point been considered for the synthesis of macroporous polymer particles by suspension polymerisation. Furthermore, suspension polymerisation has not been considered to be a suitable polymerisation technique for cationic polymerisations, since it is carried out in the presence of water, and cationic polymerisations are known to be very sensitive to moisture.

However, the present inventor has shown that by using divinyl ethers as cross-linkers in a method which is initiated by free radical chemistry and wherein a polyether solvent is used; such particles can successfully be prepared. Furthermore, the particles produced according to the invention have been shown to exhibit a novel kind of selectivity pattern when used as matrices in chromatographic separation methods, as will be illustrated in the experimental part below.

Accordingly, the use of an inert ether solvent in suspension polymerisation of divinyl ethers has not been suggested before the present invention. In this context, it is understood that the solvent is not capable of actively participating in the polymerisation process other than by dissolving the reactive monomers. Thus, the inert solvent used in the present method does not comprise any vinyl groups, i.e. it does not comprise any polymerisable carbon-carbon double bonds. Accordingly, the present invention clearly differs from the above-discussed Hirayama et al, wherein two reactive monomers but no solvent are used. Further, in conventional methods for polymerisation of polymer particles used as matrices in chromatography, such as DVB and styrene, solvents such as toluene, heptane, hexanol and other low molecule weight compounds have been used. In order to increase the pore size, it has been suggested to add smaller amounts of polymers to such conventional solvents, but the mixtures so obtained have been composed to a substantial extent of small molecular weight components. These low molecule weight compounds or mixtures have been a convenient tool to obtain a desired pore size. The present invention shows for the first time that use of a relatively high molecule weight solvent comprising ether groups can advantageously be used to produce macroporous cross-linked polymer particles from divinyl ethers. Thus, in one embodiment, the present method uses a polymeric solvent, wherein the average molecular weight of the polymer is above at least 60 g/mole, e.g. above about 100 g/mole, or at least above about 1000 g/mole.

In another embodiment, the polyether solvent is selected from the group that consists of poly(ethylene glycol) (PEG), polypropylene glycol (PPG) and poly (tetrahydrofuran). All

these solvents are obtainable from commercial sources, such as Aldrich. In an advantageous embodiment, the present method uses polypropylene glycol (PPG) as a solvent. In this context, it should be understood that the present invention also encompasses any mixture of two or more of the above-described solvents.

As regards the divinyl ether monomers used for the present polymerisation, in one embodiment, they are one or more linear, branched or cyclic alkyl, aryl or polymeric divinyl ether monomers. Thus, the divinyl ethers used according to the present invention can be characterised by the chemical formula (II)

$$C = C - O - R - O - C = C \qquad \text{(II)}$$

wherein R can be any group that does not interfere chemically or sterically with the polymerisation. Accordingly, some illustrative examples of suitable R groups are a linear, branched or cyclic alkyl or aryl chain or polymer, such as ethyl, butyl, hexyl or polyethylene glycol, polypropylene glycol or polytetrahydrofuran.

In a specific embodiment, the divinyl ether monomers are selected from the group that consists of cyclohexanedimethanol divinyl ether, butanediol divinyl ether or diethyleneglycol divinyl ether. As the skilled person in this field will realise, the choice of divinyl ether monomers will affect the nature of the polymer particle. Accordingly, if a hydrophobic particle is desired, such as for RPC, then cyclohexanedimethanol divinyl ether is selected, while if a more hydrophilic particle is desired, then diethyleneglycol divinyl ether is preferably used.

Many divinyl ether monomers that are useful in the present polymerisation are commercially available, e.g. cyclohexanedimethanol divinyl ether, butanediol divinyl ether or diethyleneglycol divinyl ether (all available from Aldrich).

Alternatively, the divinyl ether monomers used as starting material can be obtained by synthesis according to well-known methods, see e.g. WO 95/13861.

As mentioned above, the present method is based on the polymerisation and cross-linking of one or more divinyl ethers. However, the monomer mixture used as starting material in the present polymerisation can also comprise other monomers, such as monofunctional monomers comprising one vinyl group and/or bifunctional monomers comprising two such vinyl groups. In one embodiment, the starting material comprises, in addition to one or more divinyl ether monomers, a monomer that comprises one vinyl group which will be able to participate in polymerisation by reacting said vinyl group and one other functional group, which is not a vinyl group. Such a non-vinyl functional group can e.g. be a hydroxyl, an amine, a chlorine, or any other group that can be useful for other purposes than forming the cross-linked polymeric structure of the present particle. Accordingly, the skilled person who uses the method according to the invention can easily decide what kind of further functionalities that are needed for each intended purpose, such as an easily accessible chemical handle for further derivatisation other than via the residual vinyl groups etc. In an alternative embodiment, the present polymerisation is performed in the presence of another monomer in addition to the divinyl ethers, which comprises one vinyl group and no other functionality. Such other monomer can for example comprise a long hydrophobic aliphatic chain or aromatic group, and including such a monomer will result in a more hydrophobic surface of the particle. Thus, in this embodiment, the present invention provides a very versatile method, that can be designed depending on the intended use of the particles produced thereby.

In one embodiment, the present method also comprises a step of modification of the surface of the macroporous particles produced as described above. In an advantageous embodiment, the surface is modified with a compound that comprises a group which is a readily derivatised functionality, such as a hydroxyl group, which is suitable if a hydrophilic particle is desired. The hydroxyl may be derivatised e.g. with glycidol. Plural molecules of glycidol may be polymerically attached to the hydroxyl by addition of boron trifluoride etherate to produce a covalent coating comprising polymer chains including plural hydroxyls. Alternatively, hydroxyls may be oxidised to produce plural carboxylic acid groups. In another embodiment, the hydroxyl may be reacted with a compound such

as an epihalohydrin, such as epibromohydrin, to produce a terminal halide on the covalent coating, which may be reacted with an amine to produce a quaternary amine. An alternative advantageous method of modifying the surface is by grafting, see e.g. International patent application PCT/SE02/02159. In the present specification, grafting of a polymer surface with a hydrophobic group, namely octadecyl vinyl ether, is described in Example 1 below. Thus, modification of the surface of a macroporous particle according to the invention is easily performed by the skilled person in this field according to any well-known method.

A second aspect of the present invention is a macroporous cross-linked polymer particle, which is comprised of cross-linked divinyl ether polymers. In a specific embodiment, the present particles are comprised of polymers of one or more further subunits than divinyl ethers. The particles according to the invention can exhibit an average particle size diameter within the interval of about 2-600 μm, such as about 2-150 μm, more preferably about 3-100 μm and most preferably in the interval of about 5-30 μm.

In a specific embodiment, the macroporous cross-linked polymer particle according to the invention has been produced as described above. Thus, more details regarding this embodiment are found above.

A third aspect of the present invention is a reversed phase chromatographic (RPC) method wherein one or more macroporous polymer particles comprised of cross-linked divinyl ethers are used to separate e.g. a protein, peptide, oligonucleotide or smaller organic molecule from a liquid. The principles of RPC are well-known to the skilled person in this field.

As appears from the above, a fourth aspect of the present invention is chemical derivatisation of macroporous polymer particles comprised of cross-linked divinyl ethers with hydrophobic or hydrophilic monofunctional vinyl compounds e.g. monofunctional vinyl ethers or styrene.

A fifth aspect of the present invention is the use of divinyl ether monomers in a suspension polymerisation initiated by radical reactions. In the preferred embodiment, the suspension polymerisation is performed in an inert solvent that comprises ether or polyether groups, as discussed in detail above.

Detailed description of the drawings

Figure 1 shows separation of 4 different peptides on macroporous cyclohexane dimetanol divinyl ether particles (Polymer 2) according to the invention (upper), which has been made more hydrophobic by chemical modification with octadecyl vinyl ether, and on the same macroporous polymer particle (Polymer 2) without derivatization (lower). Polymer 2 according to the invention was prepared as described in example 1 below.

The chromatographic evaluation was performed according to example 3. In the upper chromatogram, from left to right, Ile^7-angiotensin III (2.96); Val^4-angiotensin III (3.29); angiotensin III (4.41) and angiotensin I (7.94) are shown, and four distinct peaks can be discerned. As clearly appears, the separation of Val^4-angiotensin III, angiotensin III and angiotensin I is excellent. However, this is not the case for the underivatised material (lower chromatogram), where Ile^7-angiotensin III, Val^4-angiotensin III and angiotensin III coelute (2.29) and are separated in retention time only from angiotensin I (4.63).

Figure 2 shows the separation of 6 proteins as described in example 3 and illustrates the difference in selectivity between cyclohexane dimetanol divinyl ether particles (Polymer 2) according to the invention (lower) and prior art DVB/styrene particles (Source™ 15 RPC, available from Amersham Biosciences AB, Uppsala, Sweden) (upper). It should be noted that the particle size of polymer 2 is larger (average diameter 25 μm) than Source™ 15 RPC (average diameter 15 μm), which results in somewhat broader peaks.

From left to right, the upper chromatogram shows ribonuclease (19.45); insulin (22.22); lysozyme (25.16); bovine serum albumin (BSA) and α-chymotrypsin (27.66); and ovalbumin (33.18), while the lower chromatogram shows ribonuclease (14.06); insulin

(18.18); lysozyme (20.43); bovine serum albumin (BSA) (22.56); α-chymotrypsin (23.75); and ovalbumin (28.36). The retention times are generally shorter for polymer 2 compared to Source 15™ RPC. This is a positive feature, since less organic solvent is required for the separation. This improves the process economy, which is especially important for large-scale separations.

Moreover, the separation pattern is clearly different between polymer 2 and Source™ 15 RPC. For example, Ovalbumin is better separated from α-chymotrypsin with Source™ 15 RPC than with Polymer 2.

However, base line separation between BSA and α-chymotrypsin is almost obtained with polymer 2, whereas these two proteins coelute with Source™ 15 RPC. Another difference is that ribonuclease and insulin are even better separated with Polymer 2 than with Source™ 15 RPC.

Figure 3A-C show the pore size distributions of three macroporous polymer particles (Polymer 1, Polymer 2 and Polymer 3, respectively) of the present invention as described in example 4. The effect on the average pore size of the average molecular weight of the polypropylene glycol can clearly be seen. A higher molecular weight polypropylene glycol results in a larger average pore size.

Moreover, the amount of pores with radii below 50 Å is extremely low, which is assumed to be highly advantageous in chromatographic applications.

EXPERIMENTAL PART

Below, the present invention will be illustrated by way of examples. However, the present examples are provided for illustrative purposes only and should not be construed as limiting the present invention as defined by the appended claims. All references given below and elsewhere in the present specification are hereby included herein by reference.

Example 1: Preparation of a macroporous polymer particles of the present invention

An organic monomer phase was prepared in a 50 mL beaker by combining the following ingredients:

10 mL 1,4-cyclohexanedimethanol divinyl ether (CHDVE) (98 %) (available from Sigma-Aldrich), 6.7 mL PPG (M_w 425, also available from Sigma-Aldrich) and 200 mg V65 was stirred for 30 minutes under a nitrogen atmosphere.

An aqueous phase was prepared in a 250 mL three-necked round-bottom flask equipped with a condenser, a mechanical stirrer and a nitrogen inlet by combining the following ingredients:
160 mL distilled water, 8 g Mowiol 40-88, poly(vinyl alcohol), 88 % hydrolysed available from Clariant) and 4.8 g NaCl (p.a.). The solution was stirred at 250 rpm while the temperature was raised rapidly to 65°C, where it was kept for five hours. The solution was then cooled over a 2 h period by air-cooling. After complete dissolution of the solid components, agitation was stopped, and the organic phase (prepared as described above) was added to the round-bottom flask.

The reaction mixture (combined organic and aqueous phases) was stirred at 540 rpm for 30 minutes under a nitrogen atmosphere. This resulted in organic droplets with an average diameter of approximately 20 μm. The round-bottom flask was then heated to 65 °C over a period of 20 minutes. The reaction mixture was kept at 65 °C for 24 h to polymerise the reactants.

After the polymerisation reaction was complete, the reaction mixture was allowed to cool to ambient temperature under a nitrogen atmosphere. The suspension was transferred to a sintered-glass funnel and the aqueous phase was filtered from the polymer. The polymer was washed with 500 mL distilled water, 500 mL ethanol and 500 mL acetone and finally 100 mL 23 % ethanol in water (v/v).

Example 2: Surface modification of macroporous polymer particles according to the invention

This example describes surface modification of the macroporous polymer particles of the present invention.

20 g of octadecyl vinyl ether (tech. 85 %, available from Aldrich) was melted in a 50 mL glass beaker at 65 °C for 30 minutes until everything was in the liquid form.

In a 50 mL three-necked round bottom flask equipped with a nitrogen inlet and a mechanical stirrer, the following ingredients were combined: 1.4 g of dry Polymer 2 (dried overnight at 65°C), 14 mL of liquid octadecyl vinyl ether and 350 mg of V-65. The flask was rapidly heated to 65°C. The reaction mixture was kept at this temperature and stirred at 250 rpm under a nitrogen atmosphere for 19 h.

The reaction mixture was allowed to cool to ambient temperature and it was subsequently transferred to a sintered-glass funnel and washed with 500 mL of methanol and 500 mL of ethanol.

Example 3: Chromatographic evaluation

This example describes the chromatographic evaluation of the macroporous polymer particles of the present invention for the separation of peptides and proteins.

Polymer samples of about 2.5 mL volume were packed into analytical steel columns (4.6 mm internal diameter and 15 cm length) and evaluated by performing two separations in aqueous solution, of four peptides and six proteins, respectively.
These tests were designed to determine if the polymer matrix allowed efficient mass transfer, to assess the capability of peptide and protein separation and to see how the selectivity differs from more conventional polystyrene/divinyl benzene polymers.

2 grams of dried polymer resin was mixed with 35 mL of ethanol and was allowed to stand for a minimum of 2 hours. The polymer slurry was then packed into a stainless steel column (available from Supelco, dimensions: inner diameter 4.6 mm, length 15 cm) by flow packing in ethanol at a linear velocity of 2150 cm/h until the pressure was stable. The quality of the column packing was confirmed by injecting a 50 μl pulse of 1 M sodium chloride solution in deionized water while flowing deionized water at a linear velocity of 361 cm/h. An ÄKTA™ explorer 10S HPLC system (Amersham Biosciences, Uppsala, Sweden) was used for all chromatographic evaluation. The efficiency (plates/meter) and asymmetry of the column was evaluated using Unicorn™3.1 software (Amersham Biosciences, Uppsala, Sweden). Target values for acceptable column packing parameters were a minimum of 6000 plates/meter and an asymmetry between 0.8 and 1.3.

Peptide separation

A solution of four peptides, angiotensin I, angiotensin III, Val^4-angiotensin III and Ile^7-angiotensin III (all available from Sigma-Aldrich, except for angiotensin III which was purchased from ICN) at a concentration of 0.125 mg/mL was prepared. The aqueous buffer (here denoted buffer A) was 10 mM potassium phosphate buffer (adjusted to pH 3.0 with potassium hydroxide). The organic modifier (buffer B) was 100 % acetonitrile, HPLC grade. The linear velocity was 361 cm/h. The column was equilibrated with 10 column volumes (CVs) of 3 % (v/v) B (1 CV = 2.49 mL). 10 μl of the sample mixture was injected at a linear velocity of 361 cm/h. A gradient from 3 % to 49 % B over 10 CV is performed, followed by 2 CV at 49 % B buffer. A gradient from 49 % to 3 % B buffer over 2 CV to restore the initial conditions, followed by reequilibration with 2 CVs 3% B buffer, is performed.

The results of the chromatography are presented in Figure 1.

Protein separation

Six proteins (ribonuclease, insulin, lysosyme, bovine serum albumin (BSA), α-chymotrypsin and ovalbumin, all available from Sigma-Aldrich) are included as target molecules in the protein separation assay.

All protein solutions are prepared as stock-solutions (10 mg/ml) that are kept in the freezer. The proteins are dissolved one by one in MilliQ-water, except for Insulin that needs acidic conditions to be dissolved (10mM phosphate buffer pH 2.0).

The aqueous phase (here denoted buffer A) consisted of 0.1 % (v/v) trifluoroacetic acid (TFA) in deionized water. The organic modifier (buffer B) was 100 % acetonitrile, HPLC grade.

The injection solutions are prepared by diluting known volumes of stock solution with buffer A to a final protein concentration of 0.5 mg / ml.

The linear velocity was 361 cm/h. The column was equilibrated with 10 column volumes (CVs) of 97 % (v/v) A (1 CV = 2.49 mL in this case). 25 μl of the protein mixture was injected at a linear velocity of 361 cm/h. A gradient from 3 % to 60 % B over 15 CV is performed, followed by 2 CV at 60 % B buffer. A gradient from 60 % to 3 % B buffer over 1 CV to restore the initial conditions, followed by reequilibration with 2 CVs 3% B buffer, is performed.

Example 4

This example describe how the pore size of the macroporous particles according to the invention can be altered using polymeric polyether porogens of different molecular weight. All of the polymers described below are synthesised using the procedure de-scribed in example 1.

Table 1

	Monomer	Porogen	% porogen (v/v)	Average pore size
Polymer 1	Cyclohexane-dimethanol di-vinyl ether	Polypropylene glycol average M.W. 425	40	130
Polymer 2	Cyclohexane-dimethanol di-vinyl ether	Polypropylene glycol average M.W. 1000	40	546
Polymer 3	Cyclohexane-dimethanol di-vinyl ether	Polypropylene glycol average M.W. 2000	40	882

CLAIMS

1. A method of producing one or more macroporous cross-linked polymer particles, which comprises polymerisation and cross-linking of divinyl ether monomers in an inert solvent that comprises ether groups, in which method the polymerisation is free radical initiated.
2. A method according to claim 1, wherein the ether groups are polyether groups.
3. A method according to claim 1 or 2, wherein the solvent is a mixture of ether and/or polyether solvent and alcoholic solvent.
4. A method according to any one of the preceding claims, wherein each polymer of the polyether solvent exhibits a molecular weight above about 60 g/mole.
5. A method according to claim 4, wherein the polyether solvent is selected from the group that consists of poly(ethylene glycol) (PEG), poly(propylene glycol) (PPG), and mixtures comprising PEG and/or PPG.
6. A method according to claim 5, wherein the polyether solvent is polypropylene glycol (PPG).
7. A method according to any one of the preceding claims, wherein the bifunctional vinyl ether monomers are linear or cyclic alkyl divinyl ether monomers.
8. A method according to claim 7, wherein the vinyl ether monomers are selected from the group that consists of cyclohexanedimethanol divinyl ether monomers, or a mixture thereof.
9. A method according to claim 7, wherein the vinyl ether monomers are selected from the group that consists of diethyleneglycol divinyl ether monomers, or a mixture thereof.
10. A method according to any one of the preceding claims, wherein the divinyl ether monomers are polymerised and cross-linked with at least one other monomer which is a bifunctional monomer comprising two vinyl groups.
11. A method according to any one of the preceding claims, wherein the divinyl ether monomers are polymerised with at least one other monomer which is a monofunctional monomer comprising one vinyl group.
12. A method according to claim 11, wherein said other monomers also comprises at least one non-vinyl functional group.

13. A method according to any one of the preceding claims, which is a suspension polymerisation.
14. A method according to any one of the preceding claims, which also includes a further step of modification of the surface of the particles produced by derivatisation of residual vinyl groups.
15. A macroporous cross-linked polymer particle, which has been produced by the method according to any one of claims 1-14.
16. A reversed phase chromatographic (RPC) method, wherein one or more macroporous cross-linked polymer particles according to claim 15 are used to separate a protein, peptide or oligonucleotide from a liquid.
17. Use of divinyl ether monomers and an inert solvent that comprises ether groups in a suspension polymerisation initiated by radical reactions.

Figure 1

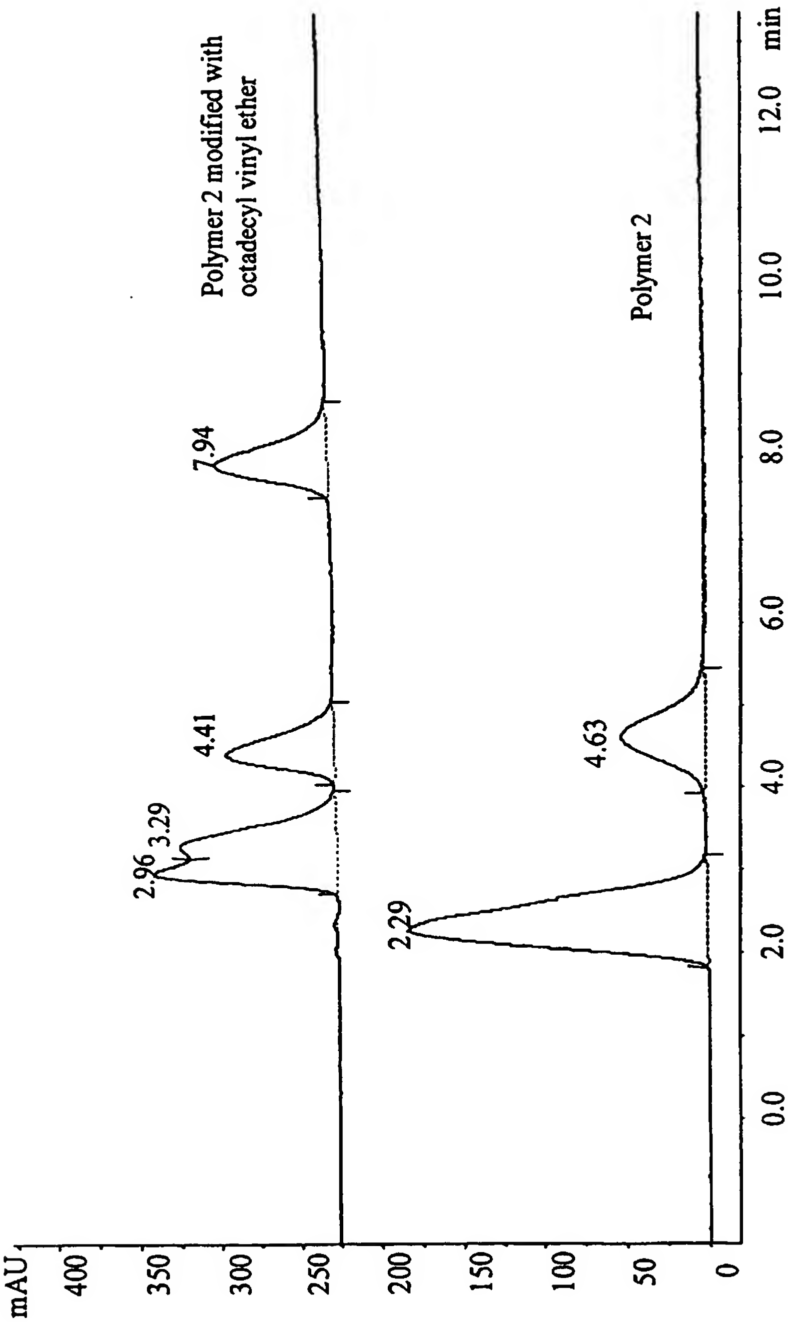

Polymer 2 modified with
octadecyl vinyl ether
Polymer 2
2.96
3.29
4.41
7.94
2.29
4.63
mAU
400
350
300
250
200
150
100
50
0
0.0
2.0
4.0
6.0
8.0
10.0
12.0
min

Figure 2

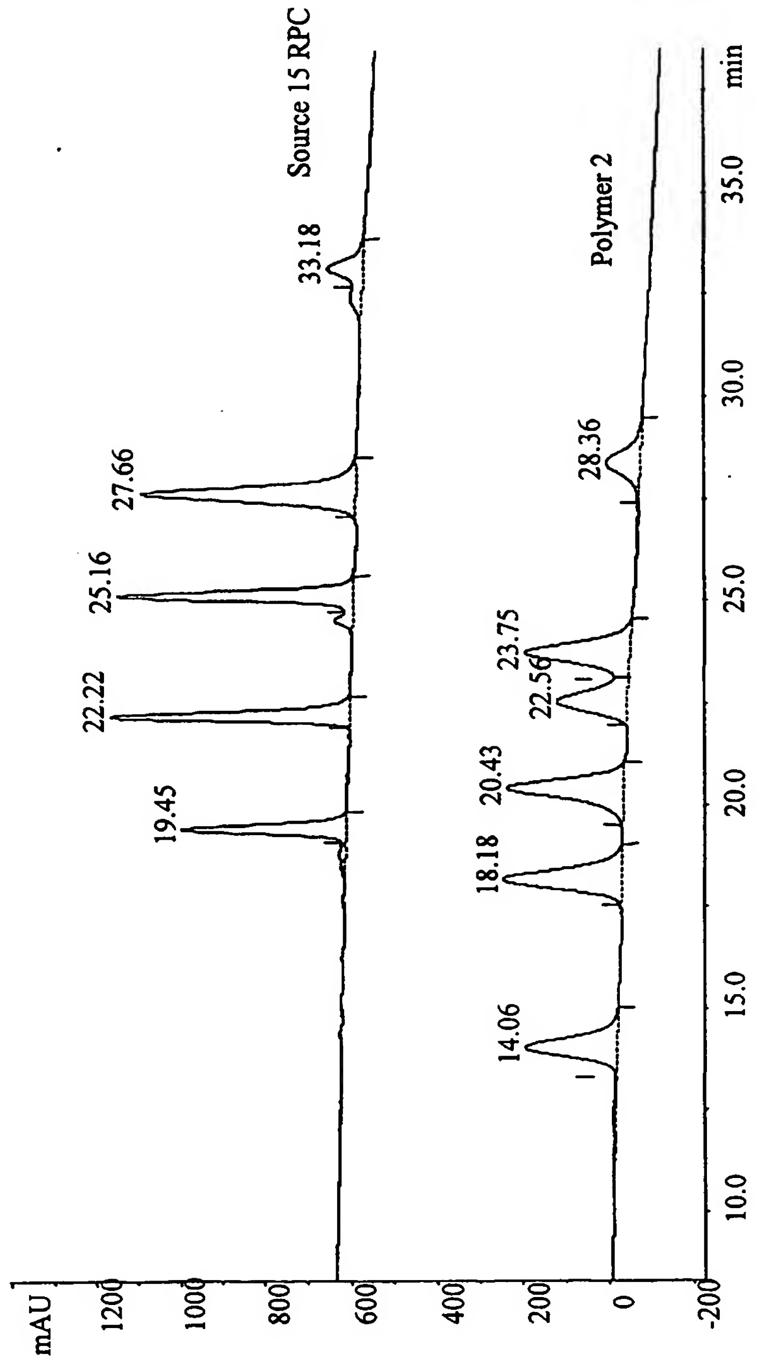

Source 15 RPC
Polymer 2
19.45
22.22
25.16
27.66
33.18
14.06
18.18
20.43
22.56
23.75
28.36
mAU
1200
1000
800
600
400
200
0
-200
10.0
15.0
20.0
25.0
30.0
35.0
min

Figure 3 A

Pore size distribution of polymer 1

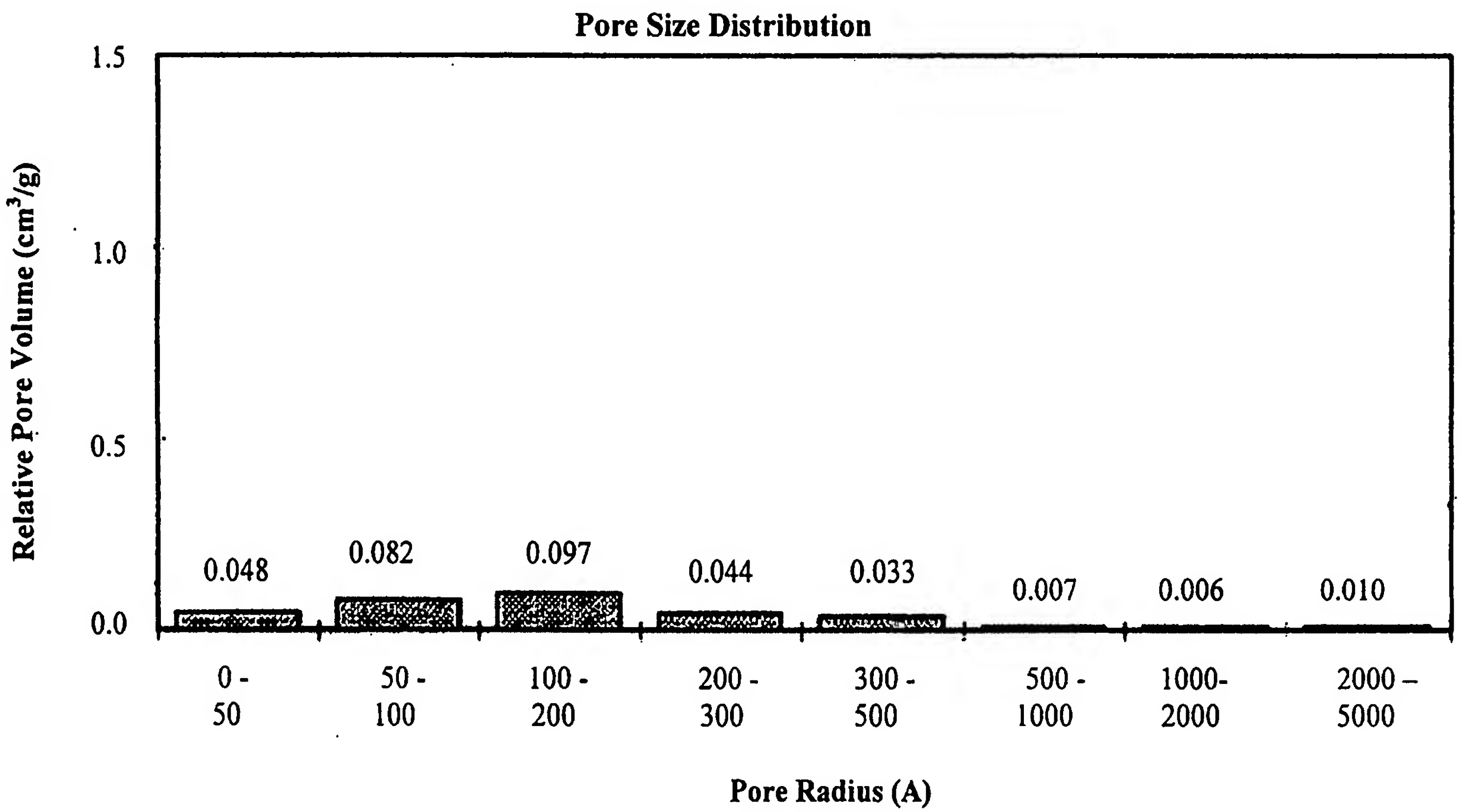


Figure 3 B

Pore size distribution of polymer 2:

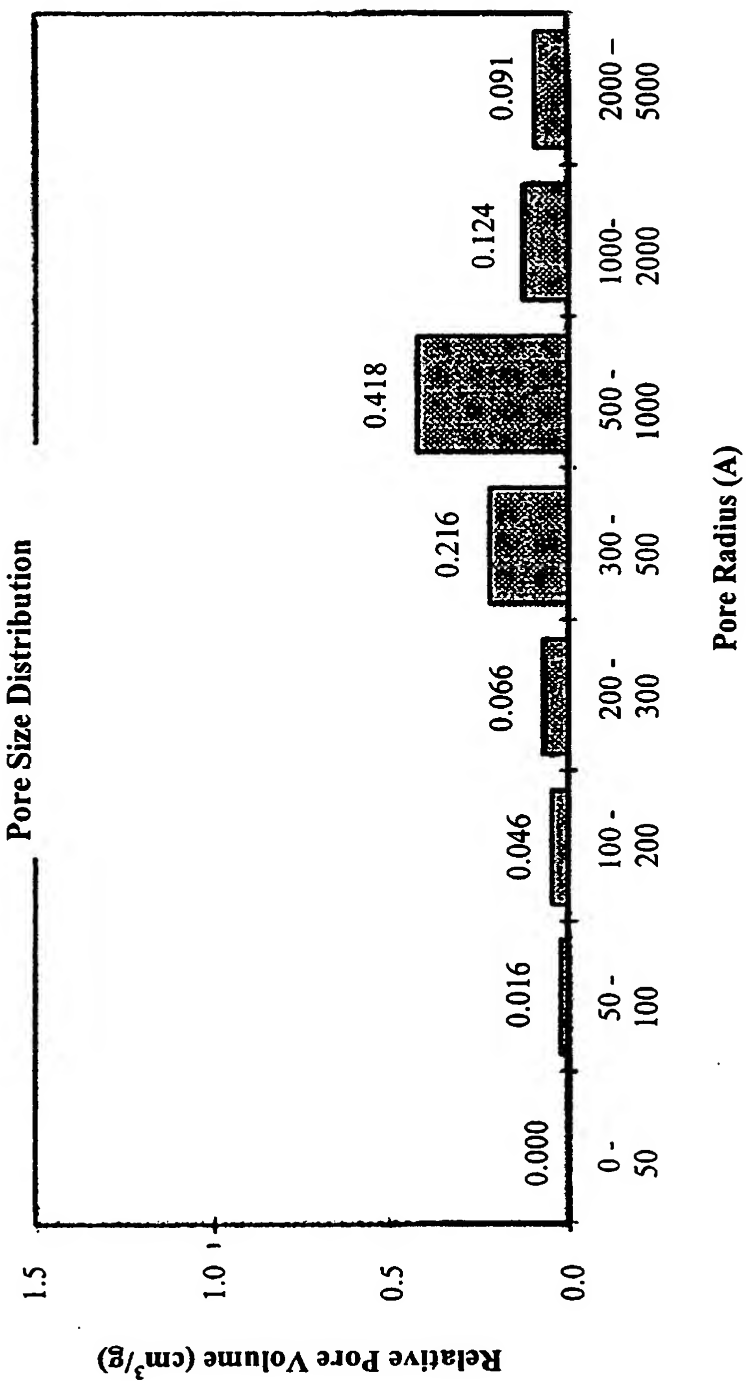
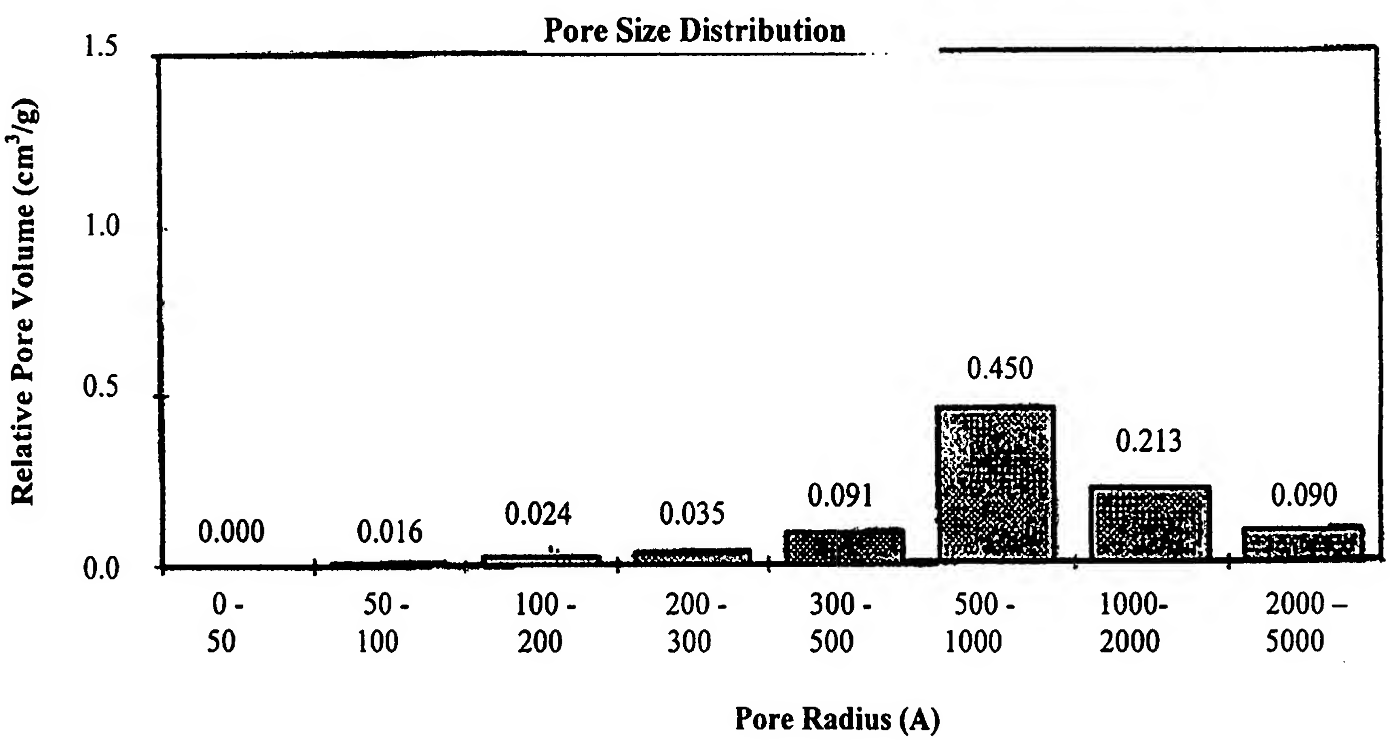


Figure 3 C

Pore size distribution of Polymer 3:



INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 03/00868

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C08F 16/32, C08F 2/18, B01D 15/08, G01N 30/48
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: B01D, C08F, G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC, WPI, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5334310 A (JEAN M.J. FRECHET ET AL), 2 August 1994 (02.08.94), column 5, line 13 - column 6, line 28; column 7, line 35 - line 64 --	1,3,11-12,14
A	Chromatographia, Vol. 33, 1992, 01, C. Hirayama et al: "Porous Polymer Packings from Vinyl Ether Derivatives for Reversed-Phase Liquid Chromatography", pages 19-24 --	1-17
A	WO 0240559 A2 (MATRIX INNOVATION INC.), 23 May 2002 (23.05.02), page 6,11	1-16
X	 --	17

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 August 2003	21 -08- 2003

Name and mailing address of the ISA/	Authorized officer
Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Barbro Nilsson/Els Telephone No. +46 8 782 25 00

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4568706 A (SIEGFRIED NOETZEL ET AL), 4 February 1986 (04.02.86), column 4, line 51 - line 58, claim 10 --	1-17
A	WO 9944053 A2 (BIA SEPARATIONS D.O.O.), 2 Sept 1999 (02.09.99), claims 5,20 -- --------	1-17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE03/00868

Box I **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

I One invention relating to a method of producing macroporous particles, the particles produced and theirs application in chromatographic methods according to claims 1-16.

II. One invention relating to the use of divinyl ether monomers and an inert solvent in a suspension polymerisation according to claim 17. The subject matter in claim 17 lacks a common technical features with I.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

26/07/03

International application No.
PCT/SE 03/00868

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5334310 A	02/08/94	US 5453185 A	26/09/95
		DE 69211010 D,T	23/01/97
		EP 0609373 A,B	10/08/94
		SE 0609373 T3	
		JP 3168006 B	21/05/01
		JP 7501140 T	02/02/95
		WO 9307945 A	29/04/93
WO 0240559 A2	23/05/02	AU 1808902 A	27/05/02
US 4568706 A	04/02/86	AT 38678 T	15/12/88
		AU 564409 B	13/08/87
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		BR 8402533 A	02/04/85
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		ES 532831 A	01/01/85
		ES 8502133 A	16/03/85
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		FI 842078 A	29/11/84
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		HU 36151 A	28/08/85
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		PT 78643 A,B	01/06/84
		ZA 8404024 A	24/12/84
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		CA 2322009 A	02/09/99
		EP 1058844 A	13/12/00
		JP 2002505428 T	19/02/02
		SI 9800058 A	31/08/99
		SI 20011 A	29/02/00